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Binding of TAFs to Core Elements Directs Promoter Selectivity by RNA Polymerase II

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Summary

The mechanisms that govern core promoter recognition and basal transcription efficiency remain poorly understood. Here, we have assessed the potential role of TAFs and the TFIID complex in directing basal promoter function. Reconstituted transcription reactions revealed the ability of TFIID versus TBP to discriminate between distinct core promoters. A comparison of different partial TBP-TAF assemblages established that a trimeric TBP-TAF$_{II}$250-TAF$_{II}$150 complex is minimally required for efficient utilization of the initiator and downstream promoter elements. Depending on the promoter structure, TAFs can increase or decrease the stability of TFIID-promoter interactions. These findings suggest that TAFs play a critical role in promoter selectivity and transcription regulation through direct contacts with core promoter elements.

Introduction

The transcriptional control regions of eukaryotic protein coding genes can be separated into at least two categories: a core promoter and upstream (or downstream) regulatory elements. Each gene carries a unique array of proximal and distal enhancer elements that are recognized by sequence-specific DNA-binding factors critical for activating or repressing transcription initiation (Tjian and Maniatis, 1994). The core promoter, on the other hand, nucleates the assembly of an initiation complex containing RNA polymerase II (pol II) and a complement of accessory factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) that can direct a low level of basal transcription in vitro, even in the absence of activators (Conaway and Conaway, 1993; Zawel and Reinberg, 1992; Buratowski, 1994). Although far from uniform, core promoters of many different RNA pol II genes show structural similarities (Weis and Reinberg, 1992; Smale, 1994). Many class II promoters contain both a TATA box (typically at position −25 to −30) and an initiator element (consensus PyPyA^{+1}NT/APyPy) overlapping the transcription start site. In the absence of a TATA box, the initiator can specify the start site of transcription. The lack of a TATA box or initiator may lead to a weakened promoter. In addition to these elements, other DNA sequences often located immediately downstream of the transcription start site can also contribute to basal promoter strength, although no clear sequence motifs have been identified. Thus, it has become increasingly evident that gene regulation is governed not only by upstream enhancers, but may also be critically dependent on the elements within core promoters.

The most extensively characterized of the core promoter recognition factors is the TATA box–binding protein (TBP) subunit of the TFIID complex. A detailed biochemical dissection of TFIID has revealed that this transcription factor consists of TBP and at least eight tightly bound subunits, the TBP-associated factors, or TAFs (Dynlacht et al., 1991; Tanese et al., 1991). TFIID is thought to be the only basal transcription factor possessing sequence-specific DNA-binding activity that is mediated by TBP (Hernandez, 1993) and possibly TAF$_{II}$150 (Verrijzer et al., 1994). Binding of TFIID to the core promoter is considered to be the first step in the assembly of an active initiation complex (Buratowski et al., 1989; Zawel and Reinberg, 1992; Conaway and Conaway, 1993; Buratowski, 1994). Until recently, most of the analysis of TFIID has focused on its essential role in mediating transcriptional regulation by upstream activators. It has been established, for example, that at least some of the TAFs can serve as coactivators that make contact with enhancer-binding proteins to direct gene-specific transcriptional activation (Goodrich and Tjian, 1994; Chiang et al., 1993; Chen et al., 1994; Jacq et al., 1994). Importantly, in the cell, TBP is found stably associated with different sets of TAFs to form the distinct initiation complexes SL1, TFIID, and TFIIIB, which selectively direct transcription by RNA pol I, pol II, and pol III, respectively (Hernandez, 1993). Apparently, the presence of distinct TAFs bound to TBP programs these transcription complexes to discriminate between promoters designated for different classes of RNA polymerases. These findings suggested that TAFs may play an important role, not only as coactivators that mediate enhancer-dependent activation, but also as core promoter recognition factors.

Despite a plethora of studies identifying components of the basal transcriptional apparatus that assemble on the core promoter, little is known about the biochemical mechanisms by which intrinsic core promoter strength is determined. For example, the identities and properties of potential regulatory factors that recognize and bind to downstream core promoter elements remain obscure. Similarly, it is unclear which transcription factors are responsible for initiator activity. Several proteins have been reported to be involved in initiator function including RNA pol II itself (Carcamo et al., 1991), transcriptional activators such as E2F (Means et al., 1992), YY1 (Usheva and Shenk, 1994), and USF (Du et al., 1993), the putative initiator factor TFII-I (Roy et al., 1993), and TFIID (Wang and Van Dyke, 1993; Kaufmann and Smale, 1994; Purnell et al., 1994; Martinez et al., 1994). Taken together, these observations imply that the mechanisms by which core promoters govern transcriptional activity may be gene specific and subject to regulation by various transcription factors including TAFs.

An important clue that TAFs in the TFIID complex may

*The first two authors contributed equally to this work.

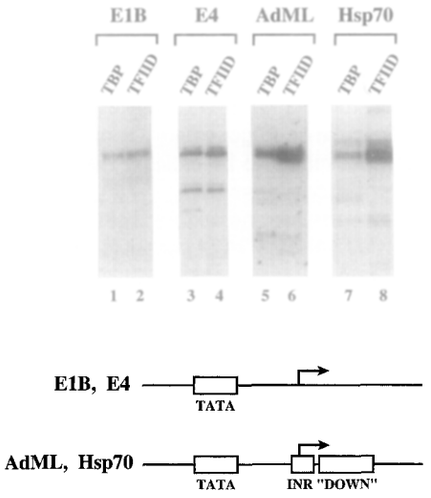

Figure 1. Differential Function of TBP and TFIID on Distinct Promoters

The ability of TBP versus TFIID to direct basal transcription was determined in a TFIID-dependent Drosophila fractionated transcription system using four different promoters, *E1b* (lanes 1 and 2), *E4* (lanes 3 and 4), AdML (lanes 5 and 6), and Drosophila *hsp70* (lanes 7 and 8). Recombinant purified Drosophila TBP was added to the odd-numbered lanes, and an equimolar amount of purified Drosophila TFIID was added to the even-numbered lanes. A schematic cartoon indicates the differences in promoter structure between the distinct promoters. Transcription products were visualized by primer extension.

contribute to core promoter function came with the finding that $TAF_{II}150$ is able to directly recognize and bind to sequences overlapping the initiator and extending to position +35 of certain promoters (Verrijzer et al., 1994). However, the functional relevance of TAF–promoter interactions for transcription initiation remained undetermined. Here, we have assessed the potential role of the pol II TAFs in core promoter function and transcriptional specificity. First, we tested the ability of purified TBP versus holo-TFIID to discriminate between different promoters. Next, the role of individual TAFs in mediating core promoter activity was tested by assaying the activity of various wild-type and mutant promoters in combination with different TBP–TAF complexes. DNA binding and template commitment experiments were carried out to determine the specificity and mechanism of TAF-dependent core promoter function. Our results reveal novel activities carried out by TAFs that direct promoter selectivity, modulate the stability of initiation complexes, and govern transcriptional regulation.

Results

TFIID but Not TBP Discriminates between Different Core Promoters

As a first step toward determining the potential role of TAFs in core promoter selectivity, we compared the ability of TBP versus TFIID to direct basal transcription from several different natural promoters. These studies were performed with two distinct classes of promoters. First, we used promoters containing a TATA box but no initiator-like sequences or further downstream core elements, represented by the adenovirus early region 1b and 4 (*E1b* and *E4*) promoters. Second, we tested promoters such as the adenovirus major late (AdML) and the Drosophila *heat shock protein 70* (*hsp70*) promoter that have a TATA box and an initiator, as well as downstream sequences recognized by TFIID. Previous studies have reported that the DNase I protection pattern of TFIID on the latter two promoters encompasses an extended region that includes the initiator and downstream regions, whereas TFIID protects only a 20 bp region centered around the TATA box of the *E1b* and *E4* promoters (Sawadogo and Roeder, 1985; Zhou et al., 1992; Chiang et al., 1993; Emanuel and Gilmour, 1993). Transcription from these four templates was carried out in a fractionated transcription system derived from Drosophila embryos supplemented with either purified Drosophila TFIID or purified recombinant Drosophila TBP. Figure 1 shows that equal molar amounts of TBP or TFIID direct comparable levels of transcription from both the *E1b* and *E4* promoters (lanes 1–4). In striking contrast, TFIID supports significantly higher levels of transcription than TBP from the AdML and *hsp70* promoters (Figure 1, lanes 5–8). These experiments, using distinct promoters, reveal a critical function of TAFs in core promoter selectivity.

Binding of the *hsp70* Core Promoter by a Trimeric TBP–TAF Complex

To test directly the functional relevance of TAFs during core promoter recognition, we set out to compare the DNA-binding and transcription properties from a wild-type promoter containing an initiator and downstream elements with those of a mutant promoter lacking these core elements. The Drosophila *hsp70* promoter appears to be ideally suited for these studies, as it showed a strong differential response between TBP and TFIID (Figure 1, lanes 7 and 8). Moreover, a number of mutant templates lacking portions of the downstream promoter elements have been described previously (Emanuel and Gilmour, 1993). As an initial step toward dissecting the potential role of TAFs in promoter recognition of the *hsp70* core elements, we carried out direct DNA binding studies with various TBP–TAF complexes. First, we used a DNA cross-linking strategy to identify specific TAF–DNA interactions in the context of holo-TFIID. Immunopurified endogenous TFIID was allowed to bind a radiolabeled *hsp70* promoter fragment (position −45 to +40) substituted with bromodeoxyuridine (BrdU). The resulting protein–DNA complexes were subjected to ultraviolet (UV) cross-linking and nuclease digestion, followed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis and autoradiography (Figure 2A). Out of the nine proteins in the TFIID complex, we detected two polypeptides with approximate molecular masses of 250 kDa and 150 kDa that became selectively labeled upon UV cross-linking (Figure 2A, lane 1). A control immunoprecipitation reaction lacking TFIID resulted in no labeled proteins detected (Figure 2A, lane 2). We next tested binding of an in vitro assembled recombinant trimeric complex (TBP–$TAF_{II}250$–$TAF_{II}150$) by UV cross-linking. Again,

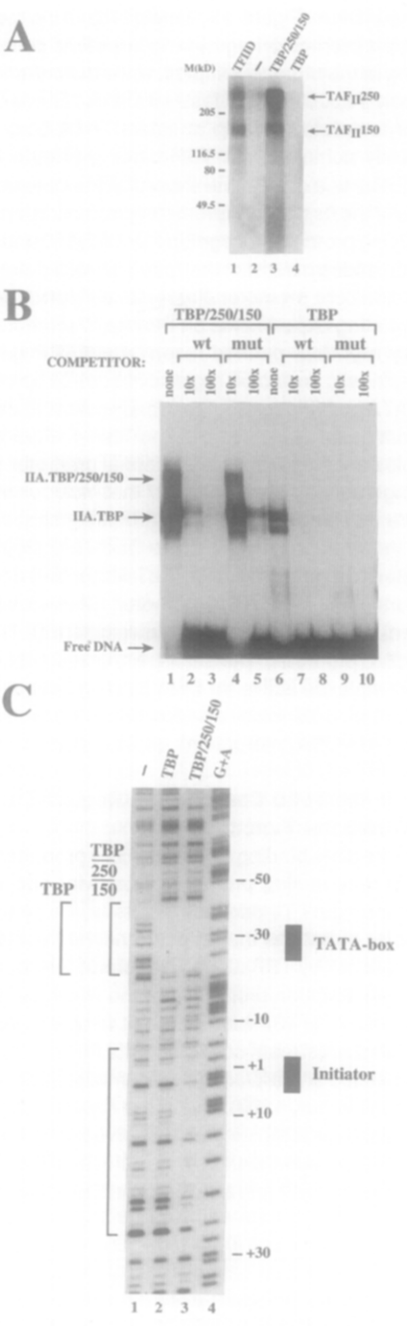

Figure 2. Recognition of Core Promoter Elements by a Trimeric TBP–$TAF_{II}250$–$TAF_{II}150$ Complex

(A) UV cross-linking of TFIID subunits to the *hsp70* core promoter. Drosophila TFIID, immunopurified with anti-$TAF_{II}80$ monoclonal antibodies (lane 1), control antibodies (anti-HA, lane 2), TBP–$TAF_{II}250$–$TAF_{II}150$ complex (lane 3), or TBP alone (lane 4) was allowed to bind a ^{32}P-bodylabeled, BrdU-substituted *hsp70* promoter fragment (position −45 to +40). After UV cross-linking and nuclease treatment, proteins were analyzed by SDS–PAGE followed by autoradiography. The positions of $TAF_{II}250$ and $TAF_{II}150$ are indicated.

(B) DNA binding of TBP–$TAF_{II}250$–$TAF_{II}150$ (lane 1) and TBP (lane 5) was compared in a bandshift experiment using a 95 bp radiolabeled *hsp70* promoter fragment (position −45 to +40). All binding reactions were in the presence of TFIIA. Binding of TBP–$TAF_{II}250$–$TAF_{II}150$ complex (lanes 1–5) or TBP alone (lanes 6–10) to the wild-type *hsp70* promoter fragment was tested in the absence (lanes 1 and 6) or presence of a 10- to 100-fold excess of cold competitor DNA corresponding to either the wild-type *hsp70* promoter (wt, lanes 2, 3, 7, and 8) or mutant *hsp70* (−3) (position −45 to +35; mut, lanes 4, 5, 9, and 10) promoter fragment.

(C) DNase I footprinting of TBP–TAF complexes on the *hsp70* promoter. All binding reactions were in the presence of TFIIA. DNase I digestion patterns on the *hsp70* promoter, radiolabeled on the transcribed strand, in the presence of no protein (lane 1), TBP (lane 2), or the TBP–$TAF_{II}250$–$TAF_{II}150$ complex (lane 3). Products were analyzed on a 8% polyacrylamide gel in parallel with G+A sequence reactions (lane 4). The locations of the fragments on the gel relative to the transcription start site are indicated, and clearly protected regions are indicated by brackets.

polypeptides corresponding to recombinant $TAF_{II}250$ and $TAF_{II}150$ became labeled (Figure 2A, lane 3). Together with previous DNA cross-linking data (Gilmour et al., 1990; Verrijzer et al., 1994; Sypes and Gilmour, 1994), these experiments lend strong support to the notion that within the native TFIID complex $TAF_{II}250$ and $TAF_{II}150$ may directly contact the promoter DNA. We suspect that TBP is not detected in these assays, because BrdU-substituted DNA only efficiently cross-links proteins that contact the major groove, while TBP contacts DNA predominantly via the minor groove (Klug, 1993).

To address the sequence specificity of the DNA–TAF interactions, we carried out both bandshift as well as DNase I protection assays using recombinant purified TBP–$TAF_{II}250$–$TAF_{II}150$ complex. Mobility shift assays were performed in the presence of TFIIA to stabilize the protein–DNA complexes during electrophoresis. We note that the presence or absence of TFIIA had no detectable effect on the promoter recognition properties of TFIID in our transcription experiments (data not shown). Using a radiolabeled *hsp70* promoter fragment containing the TATA box as well as downstream elements (position −45 to +40), we found that the TBP–$TAF_{II}250$–$TAF_{II}150$ complex produces a distinct bandshift that migrates considerably slower than the TBP–IIA–DNA species (Figure 2B, lanes 1 and 6). In binding reactions containing the trimeric TBP–TAF complex, we observed two major shifts: one corresponding to a TBP–IIA–DNA complex and a broader, slower migrating species corresponding to the TBP–IIA–$TAF_{II}250$–$TAF_{II}150$–DNA complex as determined by antibody supershift experiments (data not shown). Since reimmunoprecipitation experiments indicate that the trimeric complex is stable in solution, we consider it likely that the presence of some free TBP in the binding reactions is due to dissociation of the trimeric complex during electrophoresis, resulting in a TBP–IIA–DNA shift and a smear between the two major retarded bands. We next assessed the contribution of the initiator and downstream sequences in the *hsp70* promoter toward binding of the trimeric complex by template competition experiments. Increasing amounts of unlabeled wild-type promoter DNA or mutant promoter DNA of similar length but lacking the downstream elements were added to binding reactions containing the trimeric complex and radiolabeled *hsp70*

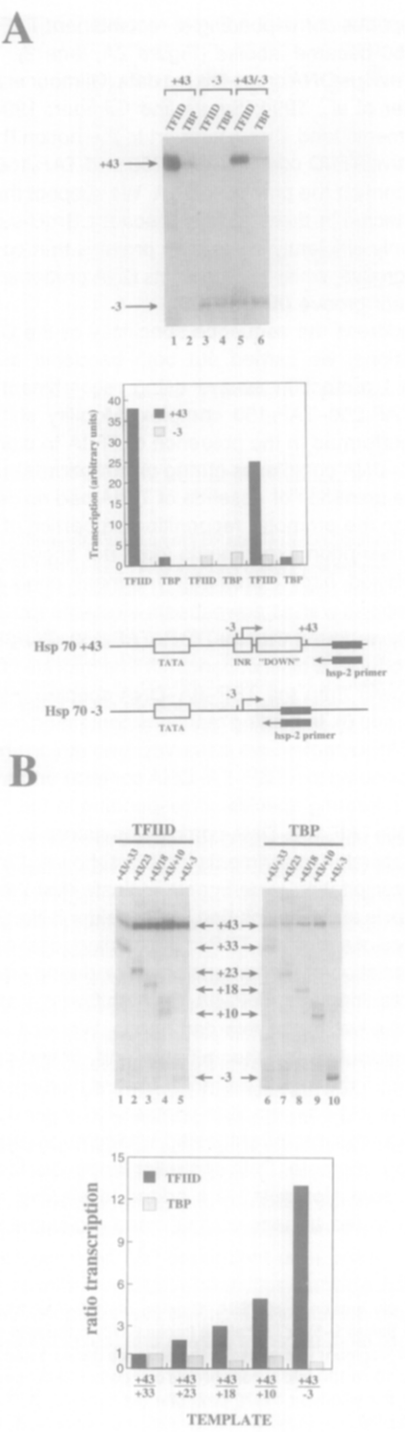

Figure 3. TFIID Is Required for Function of Downstream Promoter Elements

(A) The function of TFIID and TBP were compared in a TFIID-dependent Drosophila fractionated transcription system. Endogenous purified Drosophila TFIID was added to the odd-numbered lanes, and recombinant purified Drosophila TBP (2 ng) was added to the even-numbered lanes. The molar amount of TBP in each reaction is approximately equal to that of TFIID. Schematic diagrams of the templates used here are shown, which include the *hsp70* (+43) template and a derivative in which the region between −3 and +43 has been deleted, *hsp70* (−3). Templates (80 ng) in the transcription reactions were *hsp70* (+43) (lanes 1 and 2), *hsp70* (−3) (lanes 3 and 4), or both templates (lanes 5 and 6). Transcription products were visualized by primer extension. Owing to the internal deletion, the primer extention products differ in length. The levels of transcription were quantitated by phosphorimager (Molecular Dynamics) and plotted.

(B) The promoter strength of a series of mutant *hsp70* promoters containing progressive 3′ deletions was compared with that of the wild-type (+43) *hsp70* promoter in the presence of either TFIID (lanes 1–5) or TBP (lanes 6–10). The 3′ deletion breakpoints are located at position +43, +33, +23, +18, +10, or −3. Each transcription reaction contained an equal amount (80 ng) of the +43 template and one of the deletion templates. The primer extension products of each template is indicated. Transcription levels were quantified by phosphorimager analysis and plotted as the ratio of transcript from the +43 versus mutant template.

wild-type DNA (Figure 2B, lanes 1–5). As expected, the wild-type promoter fragment is a potent competitor for binding of the trimeric complex, while the mutant template competed about 10-fold less efficiently. Since both competitor templates contain an intact TATA box, they both efficiently competed for TBP binding (Figure 2B, lanes 6–10). These experiments show that the downstream elements of the *hsp70* core promoter play an important role in specifying promoter recognition by $TAF_{II}150$ and $TAF_{II}250$.

As an additional test of the specificity of binding between TAFs and core promoter elements, we performed DNase I footprinting experiments on the *hsp70* promoter. As previously reported, purified recombinant TBP generates a characteristic protected region of about 20 bp centered at the TATA box (Figure 2C, lane 2). By contrast, the DNase I footprint pattern obtained with the TBP–$TAF_{II}250$–$TAF_{II}150$ complex encompasses an additional promoter region extending from positions −4 to +27 that includes the initiator (Figure 2C, lane 3). These DNA binding studies indicate that, in contrast with TBP, the TBP–$TAF_{II}250$–$TAF_{II}150$ complex can recognize both the initiator and downstream sequences in the *hsp70* promoter. These observations confirm and extend our previous results with $TAF_{II}150$ on the AdML promoter. The availability of recombinant TBP–TAF complexes active for DNA binding puts us in a good position to determine the functional consequences of these TAF–DNA interactions on transcription initiation.

TAFs in the TFIID Complex Are Required to Mediate Core Promoter Functions

What role does binding of TAFs to core promoter elements play in determining promoter selectivity and basal promoter strength? To address this issue, we needed to find promoter mutations that affect transcription mediated by TFIID but not by TBP alone. Therefore, we compared the basal transcription properties of the wild-type *hsp70* promoter, *hsp70* (+43), with a mutant template, *hsp70* (−3), in which downstream core elements from −3 to +43 have been deleted and replaced by plasmid sequences (Emanuel and Gilmour, 1993). Using the *hsp70* (+43) promoter as a wild-type template, we observed that transcription with TFIID is substantially more efficient than with TBP (Figure 3A, lanes 1 and 2). Moreover, TBP directed compa-

rable levels of transcription from both the wild-type promoter (Figure 3A, lane 2) and the truncated template (lane 4). In striking contrast, when TFIID is used, initiation of transcription from *hsp70* (+43) is much more efficient (approximately 15-fold) than from *hsp70* (−3) (Figure 3A, compare lanes 1 and 3). This same differential transcriptional activity between TFIID and TBP is also observed when both wild-type and mutant templates are present in the same reaction (Figure 3A, lanes 5 and 6).

To assess the contribution of the downstream core elements in the *hsp70* promoter further, we compared transcription directed by the wild-type template with a series of mutant templates lacking progressively larger portions of the downstream sequences (Emanuel and Gilmour, 1993). Deletion of sequences between +43 and +33 has no appreciable effect on core promoter function in the presence of either TFIID or TBP (Figure 3B, lanes 1 and 6). However, removal of ten additional base pairs to position +23 impairs core promoter function when transcription is directed by TFIID (Figure 3B, lane 2), while little change is seen with TBP (lane 7). Additional truncations deleting the initiator element further impaired core promoter function that is TFIID dependent (Figure 3B, lanes 3–5 and 8–10). These experiments indicate that promoter sequences located between +33 and −3 of *hsp70* contribute significantly to basal promoter function. More importantly, transcription mediated by these downstream core elements appears to require TAFs present in the TFIID complex, since TBP failed to discriminate between the wild-type and truncated templates lacking these downstream elements.

$TAF_{II}250$ and $TAF_{II}150$ Are Required for Promoter Selectivity

The binding of TAFs to core promoter elements and the differential ability of TFIID to mediate basal transcription directed by wild-type and mutant *hsp70* promoters suggested that one or more of the TAFs might serve as a promoter selectivity factor. To investigate this possibility and to determine which of the TAFs are responsible for promoter selection, we compared the transcription properties of recombinant holo-TFIID with those of various partial TBP–TAF complexes as well as with TBP alone. First, we assembled and purified two different dimeric complexes (TBP–$TAF_{II}250$ and TBP–$TAF_{II}150$) and a trimeric complex (TBP–$TAF_{II}250$–$TAF_{II}150$). We also built holo-TFIID from its nine recombinant subunits (Figure 4A). Next, we assayed the ability of these TBP–TAF complexes to discriminate between the wild-type and mutant *hsp70* core promoter templates (Figure 4B). In vitro transcription reactions supplemented with the dimeric TBP–TAF complexes revealed that these behaved essentially like TBP and were unable to mediate the function of the downstream core promoter elements (Figure 4B, lanes 1–9). By contrast, the trimeric complex containing TBP–$TAF_{II}250$–$TAF_{II}150$ directed the transcription of the *hsp70* (+43) template 10- to 20-fold more efficiently than the truncated template (Figure 4B, lanes 10–12). The differential transcription activity displayed by the trimeric complex was very similar to that observed with recombinant holo-TFIID (Figure 4B, lanes 13–15). We also tested a variety of other TBP–TAF combinations and found that the presence of both $TAF_{II}150$ and $TAF_{II}250$ is essential for promoter discrimination, while the presence of other TAFs failed to contribute significantly to core promoter recognition (data not shown). It is im-

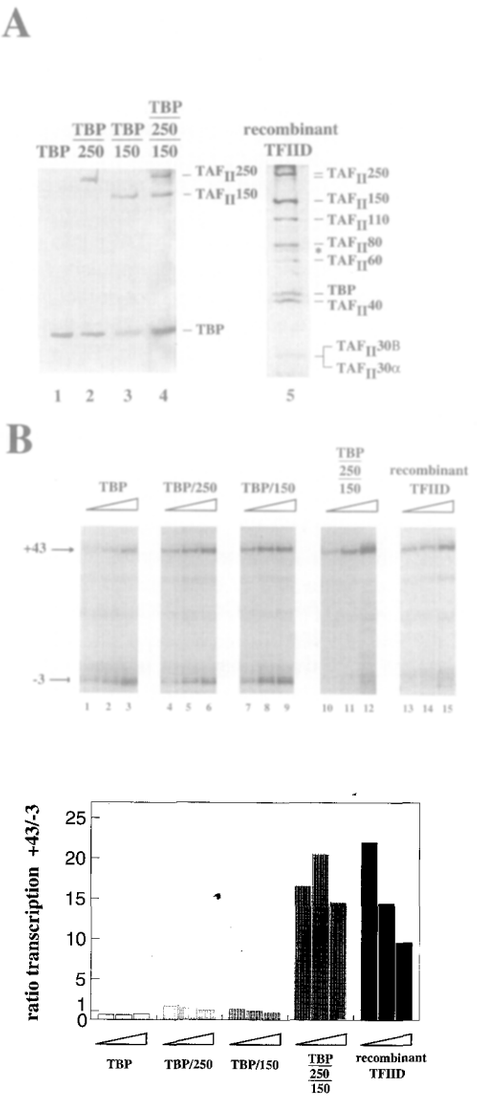

Figure 4. A TBP–$TAF_{II}250$–$TAF_{II}150$ Complex Mediates the Activity of Downstream Promoter Elements

(A) A silver-stained gel of partial TFIID complexes consisting of TBP (lane 1), TBP–$TAF_{II}250$ (lane 2), TBP–$TAF_{II}150$ (lane 3), TBP–$TAF_{II}250$–$TAF_{II}150$ (lane 4), as well as recombinant holo-TFIID (lane 5). All TFIID subunits and a $TAF_{II}80$ breakdown (asterisk) are indicated.

(B) TFIID-dependent fractionated transcription reactions containing both the *hsp70* (+43) and the *hsp70* (−3) templates (80 ng each) were supplemented with increasing amounts of TBP (about 2, 5, or 15 ng; lanes 1–3), or approximately equimolar amounts of either TBP–$TAF_{II}250$ (lanes 4–6), TBP–$TAF_{II}150$ (lanes 7–9), TBP–$TAF_{II}250$–$TAF_{II}150$ (lanes 10–12), or recombinant TFIID (lanes 13–15). Transcripts were detected by primer extension, and the products from either the +43 or −3 promoter are indicated. Transcription levels were quantified by phosphorimager analysis and plotted as the ratio of transcription from the +43 template versus −3 promoter.

portant to note that although $TAF_{II}150$ can directly bind to the core promoter, either by itself or in a dimeric TBP–$TAF_{II}150$ complex (Verrijzer et al., 1994; data not shown), transcriptional specificity was not observed. Thus, DNA binding by itself is not sufficient, and promoter discrimination apparently requires the presence of $TAF_{II}250$ in a partial TFIID complex. Moreover, we found that promoter selectivity properties of the trimeric complex, containing $TAF_{II}250$, $TAF_{II}150$, and TBP, are critically dependent on stable complex formation, since transcription reactions supplemented with all three components as free subunits failed to display differential template recognition (data not shown). A similar requirement for stable complex formation was previously observed for TAFs acting as coactivators in mediating activator-driven transcription (Chen et al., 1994). Thus, preassembly of the TAFs into a stable complex with TBP appears to be a prerequisite for TFIID function. It is noteworthy that we do not detect inhibition of transcription or DNA binding by $TAF_{II}250$ if this subunit is present in a stoichiometric complex with TBP, in contrast with a previous report (Kokubo et al., 1994). Instead, our results indicate that a TBP–$TAF_{II}250$–$TAF_{II}150$ complex is necessary and sufficient to recapitulate core recognition of the *hsp70* promoter by TFIID and to mediate transcriptional enhancement by the initiator and further downstream promoter sequences.

A TBP-$TAF_{II}250$-$TAF_{II}150$ Complex Also Mediates TdT Initiator Function

Thus far, we have determined that utilization of downstream core elements in the *hsp70* promoter requires TAFs. To extend our studies, we have assayed the ability of TAFs to recognize and mediate transcription of a different core promoter. For these experiments, we chose the well-characterized synthetic promoter containing the TATA box from the AdML promoter and the initiator from the terminal deoxynucleotidyl transferase (TdT) gene (Smale et al., 1990). As expected, TFIID directs efficient basal transcription from the template containing both a TATA box and initiator, whereas TBP supports only a low level of transcription from this template (Figure 5, lanes 1 and 3). Deletion of the initiator element (from −2 to +4) resulted in a dramatically lower (over 10-fold) level of transcription in reactions supplemented with TFIID (Figure 5, compare lanes 1 and 2). By contrast, deletion of the initiator had no appreciable effect on transcription directed by TBP (Figure 5, lanes 3 and 4). Next, we tested the transcription properties of the various partial TBP–TAF complexes with these two templates. Similar to our results with the *hsp70* promoters, the dimeric complexes TBP–$TAF_{II}150$ and TBP–$TAF_{II}250$ functioned like TBP alone, and deletion of the initiator had no effect on the level of transcription. In contrast, the trimeric TBP–$TAF_{II}250$–$TAF_{II}150$ complex, like TFIID, supported a strong transcriptional response (greater than 10-fold) to the presence of the initiator element (Figure 5, lanes 9 and 10). These experiments suggest that $TAF_{II}250$ and $TAF_{II}150$ are important for initiator function.

TAFs Can Stabilize or Destabilize TFIID-Promoter Complexes in a Sequence-Dependent Manner

We next tested the effect of TAF–promoter interactions on the stability of the transcription complex and intrinsic promoter strength. To address these questions, we have performed template commitment/challenge experiments. In these assays, a limiting amount of TFIID, TBP, or the trimeric TBP–$TAF_{II}250$–$TAF_{II}150$ complex is first preincubated with one template. Prior to the addition of the remaining basal transcription factors and nucleoside triphosphates necessary to initiate transcription, a second template is added to the preincubation mix to serve as a challenging template. If the initial protein–DNA complex has a slow dissociation rate, the second template will be largely excluded from the transcription reaction. Conversely, if the preformed initial template complex has a fast dissociation rate, the second template will compete for the limiting amounts of basal factors and be transcribed. When equal amounts of two distinct templates that have wild-type core promoters (*hsp70* [+43] and [+33]; see Fig-

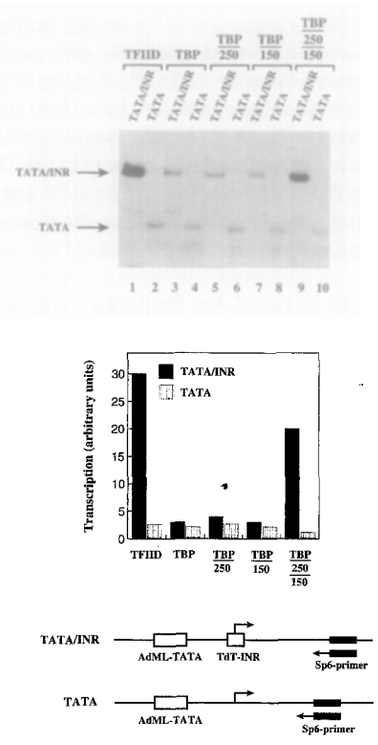

Figure 5. The Trimeric TBP–$TAF_{II}250$–$TAF_{II}150$ Complex Is Critical for Initiator Activity

The requirements for initiator function were determined by comparing transcription from two distinct templates. The TATA/INR template (odd lanes) contains a synthetic promoter consisting of the AdML TATA box fused to the TdT initiator element; the TATA template (even lanes) lacks the initiator element but is otherwise identical. Transcription reactions were supplemented with either TFIID (lanes 1 and 2), TBP (lanes 3 and 4), TBP–$TAF_{II}250$ (lanes 5 and 6), TBP–$TAF_{II}150$ (lanes 7 and 8), or TBP–$TAF_{II}250$–$TAF_{II}150$ (lanes 9 and 10). Transcription products were detected by primer extension, and transcripts from TATA/INR or TATA template are indicated. Levels of transcription were quantified by phosphorimager analysis and plotted.

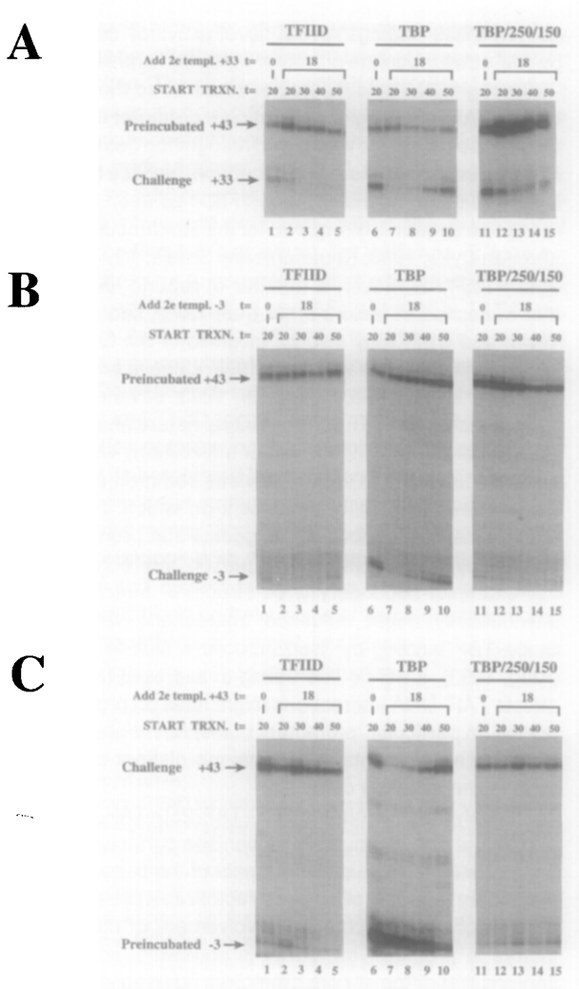

Figure 6. $TAF_{II}250$ and $TAF_{II}150$ Can Modulate the Stability of the TFIID Promoter Interactions

(A) The stability of the TFIID (lanes 1–5), TBP (lanes 6–10), and TBP–$TAF_{II}250$–$TAF_{II}150$ complex (lanes 11–15) promoter interactions were compared by template commitment/challenging assays. These factors were incubated under transcription conditions at 30°C in the presence of either both the *hsp70* (+43) template and *hsp70* (+33) template (lanes 1, 6, and 11) or only the +43 template (lanes 2–5, 7–10, and 12–15). After 18 min preincubation, the +33 template was added to the reactions lacking this template. Transcription was started either immediately (lanes 1, 2, 6, 7, 11, and 12), after 10 min (lanes 3, 8, and 13), 20 min (lanes 4, 9, and 14), or 30 min (lanes 5, 10, and 15) by the addition of the remaining transcription factors and nucleoside triphosphates at 1 min intervals. Transcription was allowed to proceed for 30 min, and transcripts were detected by primer extension. Products from the preincubated (+43) and challenging template (+33) are indicated.

(B and C) Similar template commitment/challenging experiments were performed with the *hsp70* (+43) as preincubated template and *hsp70* (−3) as competing template (B), or vice-versa, with *hsp70* (−3) as the preincubated template and *hsp70* (+43) as the competing template (C).

ure 2B) were preincubated for 20 min in the presence of either TFIID, TBP, or the trimeric complex, transcription from each template was observed to be equally strong (Figure 6A, lanes 1, 6, and 11). In contrast, when only the *hsp70* (+43) template is preincubated and then the *hsp70* (+33) template is added immediately prior to the start of transcription, the latter template is largely excluded from transcription (lanes 2, 7, and 12). Thus, TBP, TFIID, or a partial TBP–TAF complex can be committed to the *hsp70* promoter, even in the absence of the other basal factors.

To determine the relative stability of these protein–DNA complexes, the preformed complex was challenged with the second template (*hsp70* [+33]) for increasing periods of time (2, 10, 20, or 30 min) before transcription was initiated (Figure 6A, lanes 1–15). Even after 30 min, the majority of the TFIID or trimeric complex remained committed to the first template and appeared to be rather stably bound to the *hsp70* (+43) promoter with an estimated half-life of about 25 min. As expected, when we first preincubated the +33 template followed by a challenge with the +43 template, stable commitment to the +33 template was observed (data not shown). TBP can also be stably committed to the *hsp70* promoter, but its estimated half-life of approximately 7 min is significantly shorter than that of the TBP–TAF complexes under these transcription conditions. This result suggests that the TAFs can contribute significantly to the stability of TFIID binding to core promoter sequences.

Since $TAF_{II}250$ and $TAF_{II}150$ have been shown to recognize and bind specific downstream core sequences of the *hsp70* promoter, we next tested the effect of deleting these sequences on template commitment. As expected, the *hsp70* (−3) template failed to efficiently compete either holo-TFIID or the TBP–$TAF_{II}250$–$TAF_{II}150$ complexes prebound to the wild-type template (Figure 6B, lanes 1–5 and 11–15). By contrast, the TBP–*hsp70* (+43) complex was significantly challenged by the truncated promoter (Figure 6B, lanes 6–10). As expected, the half-life of TBP on either the wild-type or mutant *hsp70* promoters was similar (Figure 6C, lanes 6–10). In stark contrast, neither holo-TFIID nor the trimeric complex bind very stably to the truncated promoter (estimated half-life of less than 2 min; Figure 6C, lanes 1–5 and 11–15). Thus, the TBP–*hsp70* (−3) complex is more stable than either TFIID or the trimeric complex bound to this truncated template. This striking result suggests that the TAFs, when in complex with TBP, may actually destabilize the interaction between TBP and a template lacking downstream sequences that bind the TAFs. A similar destabilization effect of the TAFs on commitment of TFIID to the *E1b* promoter was also observed (data not shown). These results suggest that $TAF_{II}250$ and $TAF_{II}150$ can modulate the stability of TFIID–promoter interactions by means of sequence-specific recognition at selected core elements and that incorrect sequences may not be neutral but actually detrimental to the formation of stable transcriptional complexes.

Discussion

The accumulated evidence to date points to the TBP–TAF subunits of the TFIID complex as central players in the control of transcription in eukaryotes. Early studies established that TFIID and in particular its TBP subunit is responsible for binding to the TATA box and directing basal levels of transcription (Conaway and Conaway, 1993; Za-

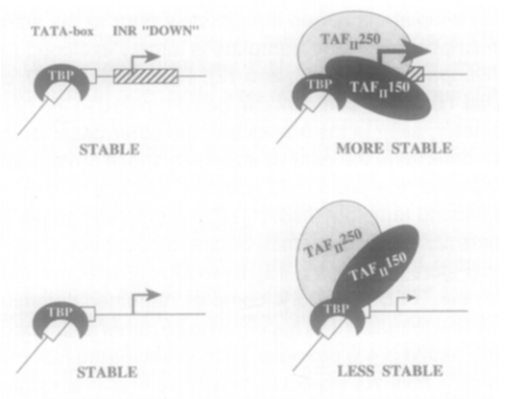

Figure 7. A Schematic Model for Core Promoter Recognition by RNA Pol II TAFs

TBP, TAF$_{II}$250, and TAF$_{II}$150 are indicated; the TATA box is represented by an open box, and the downstream regions of the core promoter, including the initiator, are represented by a hatched box. The arrow indicates the transcription start site. TBP can bind stably to the TATA box and support equal levels of transcription either in the absence or presence of downstream core promoter elements. In contrast, the TAFs in TFIID or a trimeric TBP–TAF$_{II}$250–TAF$_{II}$150 complex can recognize these core promoter sequences, increasing the stability of binding, and mediate a higher level of transcription. However, in the absence of the downstream elements, the TAFs can decrease the stability of TFIID–promoter interactions, resulting in a reduced level of transcription.

wel and Reinberg, 1992). Recent studies found that the TAF subunits of TFIID can act as coactivators that mediate transcritional activation by upstream enhancer-binding factors (Goodrich and Tjian, 1994; Chen et al., 1994). Here, we have provided direct evidence that at least some TAFs also play a critical role in core promoter selectivity by RNA pol II.

Using a combination of reconstituted transcription reactions as well as DNA binding studies, we determined that TAF$_{II}$250 and TAF$_{II}$150 can confer promoter selectivity by interacting specifically with proximal downstream elements that include the initiator. Our data suggest a close relationship between the organization of core promoter elements and the subunit architecture of TFIID. Indeed, the various core promoter elements can be viewed as an array of binding sites for distinct TFIID subunits. Depending on the precise arrangement and sequence in the core promoter, the TAFs can stabilize or destabilize the DNA binding of TFIID and modulate the preinitiation complex formation and intrinsic promoter strength (Figure 7). The surprising finding that TAFs can destabilize the binding of TFIID at certain promoters suggests that they might compensate for the rather low sequence specificity of TBP alone (Hahn et al., 1989) and prevent transcriptional initiation from occurring at weak or cryptic promoters in the genome. Thus, the function of TAFs such as TAF$_{II}$250 and TAF$_{II}$150 in discriminating between different RNA pol II promoters is reminiscent of the prokaryotic σ and Δ factors that direct promoter selectivity and prevent Escherichia coli RNA polymerase from initiating at incorrect sequences (Helmann and Chamberlin, 1988). Taken together, these findings reveal novel activities carried out by TAFs that modulate promoter utilization and selectivity.

Although DNA binding studies established the ability of certain TAFs to recognize select core promoter elements, the precise sequence motifs and recognition determinants have not been identified. The exception might be the rather loose initiator consensus, since TFIID has been shown to recognize sequences required for initiator function (Wang and Van Dyke, 1993; Kaufmann and Smale, 1994; Purnell et al., 1994). However, a number of reports have found that transcription factors other than TAFs, such as YY1, TFII-I, and USF, may also be involved in the function of specific initiators (Means et al., 1992; Usheva and Shenk, 1994; Du et al., 1993; Roy et al., 1993: Javahery et al., 1994; Smale, 1994) Thus, the general nature and specificity of initiator–transcription factor interactions will require further analysis. The lack of obvious common sequence motifs between distinct promoters on which TFIID produces an extended footprint suggests that promoter recognition by the TAFs may rely in part on "indirect readout." For example, secondary DNA structure, bendability, or unwindability might influence interactions with TFIID. Since TBP binding by itself induces a kink in the DNA (Klug, 1993), it will be interesting to see what differential effects TAF–DNA interactions might have on promoter topology. In particular, promoters that either contain or lack TAF-binding elements might assume distinct conformations in the initiation complex.

It is likely that the trimeric TBP–TAF$_{II}$250–TAF$_{II}$150 complex would provide multiple recognition surfaces that can interact with additional components of the transcriptional machinery including other basal factors as well as subunits of RNA pol II. The potential involvement of other basal factors in promoter selectivity is consistent with recent findings that different core promoters may require distinct basal factors for efficient transcriptional initiation, at least in vitro (Parvin et al., 1992; Tyree et al., 1993; Parvin and Sharp, 1993; Holstege et al., 1995). It is also conceivable that RNA pol II subunits as well as some of the other basal factors that make up the initiation complex can directly contact specific promoter elements (Carcamo et al., 1991; Maldonado et al., 1990; Coulombe et al., 1994). Thus, although TFIID may be the most critical transcription factor for recruiting and positioning RNA pol II at core promoters, multiple weak protein–DNA and protein–protein interactions between various components of the basal machinery may collectively contribute to the specificity and efficiency of transcriptional initiation.

Our finding that TAF$_{II}$250 and TAF$_{II}$150 play a role in promoter selectivity is likely to reflect a more general function of TAFs in directing transcription by the three different RNA polymerases. For example, the TAFs in SL1, TFIID, and TFIIIB may govern binding to class-specific promoters, thus preventing their recognition of heterologous templates in much the same way that TAF$_{II}$250 and TAF$_{II}$150 discriminate between different RNA pol II promoters. The multitude of functions attributed to TFIID, such as coactivator, promoter recognition, and RNA pol II recruitment, all point to a pivotal role for TFIID in receiving, integrating,

and relaying a mosaic of molecular signals imparted by the enhancer-binding proteins that regulate gene-specific transcription. The studies described here reveal novel activities associated with the subunits of TFIID, but at the same time raise additional questions concerning mechanisms of transcriptional control. A critical issue that remains to be addressed is whether differences in core promoter structure will profoundly affect the response of genes to upstream activators. For instance, it may be highly instructive to determine what role the downstream core element recognition by TAFs will play in TATA-less promoters. Is the ability of TAFs to bind core promoter elements a prerequisite for mediating transcriptional activation? Do sequence-specific interactions between core elements and TFIID trigger conformational alterations in the initiation complex necessary for recruitment of RNA pol II and its associated basal factors? Are intrinsic differences in promoter strength exploited by organisms to effect developmental and cell type–specific regulation of gene expression? With the reagents now available to reconstruct and dissect the initiation complex in detail, it may be possible to address some of these questions, and future research may unravel unexpected intricacies of transcriptional regulation in eukaryotes.

Experimental Procedures

Protein Procedures

All recombinant TFIID subunits were expressed in Sf9 cells using the baculovirus expression system. Most constructs have been described before (Chen et al., 1994). The construct expressing FLAG-tagged $TAF_{II}150$ (pVL1392-F-TAF150) was constructed by creating a NdeI site at the initiating methionine codon of the cDNA with a PCR-based strategy and subcloning of the complete coding region into a pVL1392 (Pharmingen) derivative containing a FLAG tag (MDYKDDDDK) encoding sequence upstream of an in-frame NdeI site (S. Lichtsteiner and R. T., unpublished data). This construct was cotransfected with BaculoGold viral DNA (Pharmingen) into Sf9 cells. All recombinant baculoviruses were plaque purified and amplified. For protein expression, Sf9 cells were typically infected at an MOI of approximately 5 and harvested 48 hr postinfection. All protein preparation and purification procedures were at 4°C or on ice using HEMG buffer (25 mM HEPES–KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol) containing 1 mM DTT, 0.2 mM AEBSF, 1 mM sodium metabisulfide, 2 μg/ml leupeptin, and 0.7 μg/ml pepstain and varying amounts of KCl. Whole-cell extracts were prepared by sonication in 0.4 M KCl–HEMG containing 0.1% NP-40. The sonicate was centrifuged at 100,000 × g, and the supernatant was divided into aliquots and stored at −80°C. Recombinant Drosophila TBP was purified by SP– and heparin–Sepharose chromatography essentially as described (Hoey et al., 1990), followed by DNA affinity chromatography over a TATA column. $TAF_{II}250$ was partially purified by DEAE–, Q–, SP–, and heparin–Sepharose column chromatography. $TAF_{II}150$ was purified over SP– and heparin–Sepharose chromatography. The other TAFs were purified as described (Chen et al., 1994). After purification, the protein fractions were dialyzed against 0.1 M KCl–HEMG. The in vitro assembly was carried out essentially as described (Chen et al., 1994) utilizing the HA epitope on $TAF_{II}250$ and a FLAG epitope on $TAF_{II}150$. For the assembly process, the TAFs were immobilized on protein A–Sepharose beads covalently conjugated with either anti-HA (12CA5; Zhou et al., 1992) or anti-FLAG (Kodak) monoclonal antibodies. Next, an excess of pure TBP was incubated with the beads, and after binding, the free TBP was removed by extensive washes. Using peptides corresponding to the appropriate epitope (HA, YPYDVPDYA; FLAG, DYKDDDDK), the resulting dimeric complexes were eluted under native conditions. To build the trimeric complex, we used a two-way assembly process in which first $TAF_{II}250$ was bound to $TAF_{II}150$ that was immobilized via its FLAG epitope. Next, the resulting dimeric complex was eluted and then immobilized on the HA epitope of $TAF_{II}250$. Subsequently, TBP was added to the complex, and the resulting trimer was finally eluted with the HA peptide. We also assembled recombinant holo-TFIID from its nine recombinant subunits as described (Chen et al., 1994). Resulting complexes were near homogeneous as judged by SDS–PAGE followed by silver staining (Figure 4A) and Western blotting (data not shown). Reimmunoprecipitations (Verrijzer et al., 1994) confirmed the integrity of the complexes (data not shown). Antibodies were affinity purified essentially as described (Dynlacht et al., 1991).

In Vitro Transcription Reactions

Templates used in transcription assays have been described: *hsp70* +43 to −3 (Emanuel and Gilmour, 1993); AdML-TdT, TATA/INR, and TATA (plasmids VII and V, respectively; Smale et al., 1990); AdML, *E1b*, and *E4* (Yokomori et al., 1994). In vitro transcription reactions were performed in a Drosophila fractionated system that was established essentially as described (Wampler et al., 1990), with the following changes. The TFIIB fraction was replaced by E. coli expressed recombinant, polyhistidine-tagged TFIIB (pET19b-TFIIB; C. P. V., unpublished data) purified to homogeneity by Ni–NTA– and SP–Sepharose chromatography. Furthermore, pure recombinant TFIIA(S/L) was added to reactions. TFIIA was expressed, purified, and assembled essentially as described (Yokomori et al., 1994), followed by an additional purification step over SP–Sepharose. All protein fractions were dialyzed against 0.1 M KCl–HEMG. In each 25 μl reaction, approximately the following amounts of each fraction was used: 0.3 μg of TFIID (Q.3), 0.2 μg of E/F (S.25), 0.25 μg of RNA pol II (Q.45), 1 ng of TFIIB, 10 ng of TFIIA(S/L), and 80 ng of template. Typically, about 2 ng of TBP was used and approximately equimolar amounts of each TBP–TAF complex (as indicated in the legends). Transcription reactions and primer extension analyses were carried out essentially as described (Kadonaga, 1990). Quantification was by phosphoimager analyses (Molecular Dynamics).

DNA Binding Assays

All DNA binding reactions were performed in DB buffer (50 mM KCl, 6.25 mM $MgCl_2$, 0.05 mM EDTA, 5% glycerol, and 0.5 mM DTT) and were incubated for 30 min at 25°C. For UV cross-linking, a ^{32}P-bodylabeled, BrdU-substituted probe, generated by PCR, containing base pairs −45 to +40 of the *hsp70* promoter was used. Proteins were incubated with probe in the presence of 0.5 μg of poly(dG–dC) in 50 μl of DB buffer. Reactions were then irradiated with UV (304 nm) light at room temperature for 10 min. Next, $MgCl_2$ and $CaCl_2$ to a final concentration of 100 mM, 2 μg of DNase I, and 1 U of micrococcal nuclease (Worthington) were added. After a 15 min nuclease treatment at 30°C, samples were analyzed by SDS–PAGE followed by autoradiography. For bandshift assays, ^{32}P-bodylabeled bandshift probes and cold competitor fragments that contained either base pairs −45 to +40 of the *hsp70* promoter (wild type) or base pairs −45 to +35 of the *hsp70* (−3) promoter (mutant) were generated by PCR. After binding in 20 μl of DB buffer containing 0.01% NP-40, approximately 0.3 fmol probe, and 100 ng of poly(dG–dC) competitor DNA, samples were loaded onto a 5% polyacrylamide gel (39:1 acrylamide:bis) containing 0.5 × Tris–glycine buffer plus 0.01% NP-40. Electrophoresis was at 180 V for 2.5 hr. For DNase I footprinting, a *hsp70* promoter fragment labeled by polynucleotide kinase on the transcribed strand SalI site at position +90 and extending upstream to a BamHI site at −190 (*hsp70* XBS; Emanuel and Gilmour, 1993) was used. Binding reactions were performed in 50 μl of DB buffer containing 2% polyvinyl alcohol, 100 ng of poly(dG–dC), and about 1.5 fmol of probe. Next, 2 μl of 100 mM $MgCl_2$, 50 mM $CaCl_2$ and 2 μl (1.25 μg) of DNase I were added. After 1 min digestion, 50 μl of STOP (.24% SDS, 24 mM EDTA, and 1 μg of calf thymus DNA) was added. After phenol–chloroform extraction and ethanol precipitation, samples were analyzed on an 8% sequencing gel.

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